Original Article

ALS1 and *ALS3* gene expression and biofilm formation in *Candida albicans* isolated from vulvovaginal candidiasis

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Abstract

Background: A cluster of genes are involved in the pathogenesis and adhesion of *Candida albicans* to mucosa and epithelial cells in the vagina, the important of which is agglutinin-like sequence (*ALS*) genes. As well as vaginitis is a significant health problem among women, the antifungal resistance of *Candida* species is continually increasing. This cross-sectional study investigates the expression of *ALS1* and *ALS3* genes and biofilm formation in *C. albicans* isolate isolated from vaginitis.

Materials and Methods: Fifty-three recognized isolates of *C. albicans* were collected from women with recurrent vulvovaginal candidiasis in Iran, cultured on sabouraud dextrose agar, and then examined for gene expression. Total messenger RNA (mRNA) extracted from *C. albicans* isolates and complementary DNA synthesized using reverse transcriptase enzyme. Reverse transcription-polymerase chain reaction (RT-PCR) using specific primer was used to evaluate the expression of *ALS1*and *ALS3* through housekeeping (*ACT1*) genes. 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide assay was performed to assess adherence capacity and biofilm formation in the isolated.

Results: Forty isolates (75.8%) expressed *ALS1*and 41 isolates (77.7%) expressed *ALS3* gene. Moreover, 39 isolates (74%) were positive for both *ALS1* and *ALS3* mRNA by the RT-PCR. Adherence capability in isolates with *ALS1* or *ALS3* genes expression was greater than the control group (with any gene expression), besides, it was significantly for the most in the isolates that expressed both *ALS1* and *ALS3* genes simultaneously.

Conclusion: The results attained indicated that there is an association between the expression of *ALS1* and *ALS3* genes and fluconazole resistance in *C. albicans*. A considerable percent of the isolates expressing the *ALS1* and *ALS3* genes may have contributed to their adherence to vagina and biofilm formation.

Key Words: Agglutinin-like sequence proteins, biofilm formation, *Candida albicans*, vulvovaginal candidiasis

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INTRODUCTION

*Candida albican*s is an opportunistic fungal pathogen which causes a broad spectrum of diseases such as oral, vagino-mucosal, and systemic infections.[1]

Vaginal candidiasis occurs in three-fourth of women during their life and *C. albicans* is the etiological agent in over 80% of the cases.[2]

C. albicans has a large number of virulence factors causing disease, including phenotypic switching, filamentation, adherence and secreted hydrolyses. Some of these pathogeneses are associated with gene families, particularly the agglutinin-like sequence (ALS) (6), secreted aspartyl proteinase, and lipase families.[3-7]

Among these, the *ALS* gene family that encodes cell wall glycoprotein is related to adherence to host surfaces. *ALS* genes are a family of adhesions recognized to play a role in adherence and early biofilm formation. Since biofilm formation contributes to drug resistance, *ALS* genes appear to be responsible for fluconazole resistance.[8-11] Each *ALS* gene has a similar three-domain structure, including a 5' domain of 1299–1308 bp that is 55–90% identical across the family; a central domain of variable numbers of tandemly are repeated copies of a 108-bp motif; and a 3′ domain that is relatively variable in length and sequence across the family.[12] Although they distribute a similar three-domain structure, sequence differences between the *ALS* proteins can be so large that the proteins may have different functions.[13] Since adherent isolate of *Candida* is more pathogenic, there is a theory that *ALS1* and *ALS3p* may be responsible for the pathogenesis of *C. albicans*.[14] The expression of each *ALS1* and *ALS3* gene can be detected by reverse transcription-polymerase chain reaction (RT-PCR) and confirmed by using this method for the analysis of *ALS* gene expression in the *C. albicans* genome that is correlated with its virulence. An RT-PCR assay investigates a specific gene expression in a variety of clinical specimens and in different host and model conditions.[15,16] The objective of our research was to determine the expression of *ALS1* and *ALS3* genes, its correlation with fluconazole resistance and finally biofilm formation in *C. albicans*, isolated from women with symptoms of recurrent vaginitis.

MATERIALS AND METHODS

Isolates

In this cross-sectional study, there were used 53 isolates of *C. albicans* obtained from women with recurrent vulvovaginal candidiasis that referred to clinical centers, Tehran, Iran. The patients were enrolled in terms of clinical symptoms of vaginitis and resistance to fluconazole therapy during the treatment process. Out of 53 patients, 57.69% would consume several antibiotics, 28.84% took an oral contraceptive, and 15.38% were diabetic. The isolates were confirmed by phenotypic and genotypic assays such as CHROM agar *Candida* and PCR-restriction fragment length polymorphism technique. Fluconazole susceptibility test had already been determined in our previous study.[17]

Reverse transcriptase-polymerase chain reaction analysis of *ALS1* and *ALS3* genes

The confirmed *C. albicans* isolates are used to analyze the expression of *ALS1* and *ALS3* genes with minor modification. Expression of *ALS1* and *ALS3* genes was analyzed in planktonic cells of *Candida* isolates. For RNA extraction, a 24-h colony on sabouraud dextrose agar (Merck) was transferred to a 1.5 ml eppendorf tube and then 200 µl of lysis buffer containing (200 mM Tris-Hcl, pH 7.5, 10 mM EDTA, 0.5 M NaCl), 200 µl of phenol-chloroform-isoamillalchol (25:24:1) and glass beads, were added, and the tube was strongly vibrate for 60 min. After centrifuging for 3 min at 5000 rpm, the supernatant was removed to a new tube and 300 µl of chloroform was added. After centrifuging, the aqueous phase was moved to a clean tube and then 1 volume of cold isopropanol were added and was reserved at –20°C for 15 min. After that, the sample was washed by 70% ethanol, 30 µl distilled water was added, and the sample was kept at –20°C.[18] The RNA samples were treated with 1U of DNaseI (Fermentas) per 10 µl of RNA at 37°C for 1 h, and then its quality checks by electrophoresis on agarose gel (Merck). The first strand complementary DNA (cDNA) was synthesized using 2-step RT-PCR kit (Vivantis, Malaysia) according to the manufacturer's instruction and directly used in RT-PCR assay. Briefly, 2 µl 10X Viva Buffer A, 18.5 µl distilled water, 1–2 µl cDNA, 0.35 µl forward primer and 0.35 µl reverse primer for each gene. In continue, the microtube put into thermocycling device: Initial denaturation step at 94°C for 5 min followed by 35 cycles consisting of denaturation (94°C for 1 min), annealing (58°C for 1 min), and extension (72°C for 1 min) were followed by a final extension step at 72°C for 3 min. Finally, 25 µl of PCR product should be run out on a 1% agarose gel. *ACT1* was used *ACT1* (actin) gene was used as a control housekeeping gene. Primers using in this study are shown in Table 1.

Biofilm formation assay

Candida biofilm formation of isolates was performed on a 96-well plate in four groups including the isolates with *ALS1*expression (Group 1), *ALS3* expression (Group 2),

Table 1: ALS1, ALS3, and ACT1 specific primers for reverse transcription-polymerase chain reaction assay

Primer name	Sequence (5'→3')	PCR product size (bP)	Accession number
ALS1-R	ACCAGAAGAAACAGCAGGTG	318	L25902
ALS1-F	GACTAGTGAACCAACAAATACCAG		
ALS3-F	CCAAGTGTTCCAACAACTGAA	184	AY223552
ALS3-R	GAACCGGTTGTTGCTATGGT		
ACT1-F	CCAGCTTTCTACGTTTCC	200	HM997110
ACT1-R	CTGTAACCACGTTCAGAC		

bp: Base pairs, PCR: Polymerase chain reaction

ALS1 and *ALS3* expression (Group 3) and those with any expression (Group 4). Eighty microliters of *C. albicans* cell suspension from each group was inoculated onto each well. The yeast cells were allowed to adhere to the bottom of the plate at 37°C for 90 min. Afterward, the unattached cells were gently washed with phosphate-buffered saline (PBS) and then added 4 ml of yeast nitrogen base (YNB) medium with 50 mM glucose. The plates were incubated at 37°C for 48 h, and the biofilm was made by *Candida*. Subsequently, the YNB medium was detached and washed with 4 ml of PBS.

3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide assay

For this, 100 µl 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml in PBS, Sigma) MTT was added to cells at 37°C for 3 h dimethyl sulfoxide (sigma) was added and the plates incubated for 15 min to stop the reaction and to dissolve the insoluble purple formazan. Optical absorbance was measured at the wavelength of 520 nm using an ELISA reader (Memmert, Germany).[19] The results were evaluated with regard to the percentage of viable cells in comparison to the negative control (isolates without *ALS* gene expression). All tests were performed in three independent experiments.

Statistics analysis

Data analysis was performed by *t*-test method with SPSS Statistics version 17, chicago. The level of statistical significance was set at $P < 0.05$.

RESULTS

In this study, the expressions of *ALS1* and *ALS3* genes were uniformly assessed on 3 µg of total RNA extract of isolates. RT-PCR showed the expression of *ALS1* in 75.3% and of *ALS3* in 77.2% of the isolates. Moreover, 73.5% of the isolates expressed both *ALS1* and *ALS3* genes (14.9% of the isolates had no expression for any *ALS1* and *ALS3* genes). Figure 1 illustrates the result of RT-PCR analysis. Of 53 isolates, 50 isolates were resistant

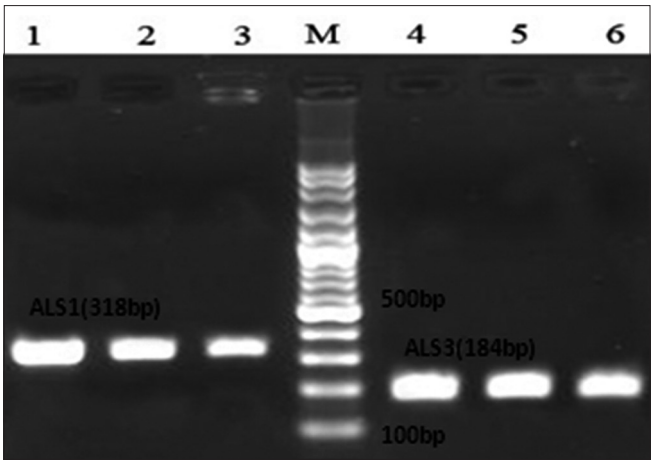

Figure 1: Messenger RNA of *Candida albicans* isolates extracted, and first strand complementary DNA was synthesized. Reverse transcriptase-polymerase chain reaction with *ALS1* and *ALS3* specific primers was done. Line 1–3, gene expression of *ALS1* in isolates (318 bp); line 4–6, gene expression of *ALS3* in isolates (184 bp). M: Marker molecular weight (100 bp)

to fluconazole (inhibition zone <12 mm), whereas 3 isolates were sensitive (inhibition zone >19 mm). The relation between fluconazole resistance with *ALS1* and *ALS3* gene expression is shown in Table 2.79% of isolates that expressed *ALS1*/*ALS3* or both genes were resistant to fluconazole.

Data from MTT assay showed the isolates with an expression of *ALS1* (Group 1) or *ALS3* genes (Group 2) had more adherence capacity than those lacking any gene expression (Group 4) and this difference was significant ($P < 0.05$). However, the isolates which had both *ALS1* and *ALS3* expressions had the highest adherence capability. Data were means ± standard deviations of three independent experiments ($P < 0.05$).

The result is shown in Figure 2.

DISCUSSION

In this study, the expressions of *ALS1* and *ALS3* genes among the *C. albicans* isolates were analyzed using RT-PCR method. The presence of the genes has been reported in our previous study.[17] The simultaneous existence of both *ALS1* and *ALS3* genes was detected in 83% of the isolates, among which 74% expressed the two genes, summarized in Table 2. Our results are consistent with what reported by Cheng *et al*.[20] that evaluated the expression of *ALS* genes in *C. albicans* isolates using RT-PCR and showed a high frequency of *ALS1*, *ALS2*, *ALS3*, and *ALS9* genes expression.

Nas *et al*.,[21] evaluated the expression of *ALS1* gene in *C. albicans* isolated from vaginal candidiasis and showed its expression in 69% of total isolates.

Table 2: Frequency of expression of *ALS1* and *ALS3* genes of *Candida albicans* isolates

Profile	Resistance/ sensitive	ALS1/ALS3 expression	Percentage
1	Resistant	−/−	5 (9.4)
2	Resistant	+/+	39 (74)
3	Resistant	+/−	1 (1.8)
4	Resistant	−/−	3 (5.6)
5	Resistant	−/+	2 (3.7)
6	Sensitive	−/−	2 (3.7)
7	Sensitive	−/−	1 (1.8)

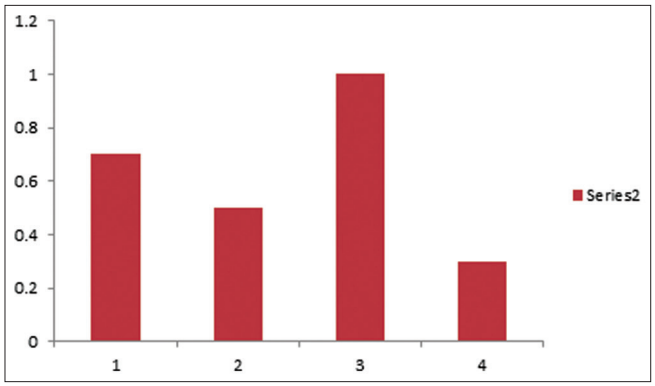

Figure 2: 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide assay: Biofilm formations in four groups of *Candida albicans* isolates in yeast nitrogen base medium containing 50 mM glucose. The method used for biofilm quantification was 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide reduction. Data were means ± standard deviations of three independent experiments ($P < 0.05$). Group 1 (isolates with *ALS1* expression), Group 2 (isolates with *ALS3* expression), Group 3 (isolates with *ALS1* and *ALS3* expression), and Group 4 (control group without gene expression)

In this study, 42 (79.5%) of isolates with an expression of at least one *ALS* gene were resistant to fluconazole and 3 (5.5%) of them without *ALS* gene expression were absolutely sensitive to fluconazole. A significant relationship was found between the *ALS1* and *ALS3* genes and biofilm formation in *C. albicans* isolates in MTT assay. Our finding hypothesized that there is a positive correlation between the expressions of *ALS1*, *ALS3* genes, and fluconazole resistance. Since all of *C. albicans* isolates expressing the *ALS1* or *ALS3* were resistant to fluconazole, and their adherence was stronger than the control group (without any expression) and it seems reasonable to conclude that biofilm contributes to fluconazole resistance in isolates. This finding is in agreement with the results of other studies according to which the *ALS1* was introduced as the major over-expressed gene in biofilm formation and adherence which in turn play an important role in drug inhibition to gain access to the fungi microcolonies.[22-24] Similar findings by other investigators which show pathogenic *Candida* species established well-developed biofilm and are resistant to drug therapy and act as a source of reinfections. More studies are required to realize the exact mechanisms involved.[25,26] According to our result, 9.4% isolates of *C. albicans* did not contain *ALS1* and *ALS3* genes, though they were resistant to fluconazole. This may occur due to other mechanisms involved in fluconazole resistance which affect phenotype. Moreover, the role of Med31, Med20, and Srb9/Med13 transcriptional regulator genes in the expression of the relevant virulence genes including *ALS* gene family and biofilm formation should not be ignored.[27]

It has been shown that *C. albicans* Tor1 gene plays a crucial role in the regulation of expression of several virulence-associated genes such as *ALS1* and *ALS3* and other adhesions. In addition, as a regulatory complex, Tec1, Bcr1, Efg1, Nrg1, and Tup1 affect the expression of several adhesions and *ALS* genes family.[28] Many other studies show the significance of regulatory genes in the expression of *ALS* gene family that explain the nonexpressed *ALS1* and/or *ALS3* genes in the isolates.[29,30]

CONCLUSION

The findings of this study demonstrate that the *ALS* genes expression contributes to adherence and fluconazole resistance in clinical isolates of *Candida* to the vagina. Regulatory network genes play a significant role in whether *ALS* genes are actively expressed or kept silence. The expression of *ALS* genes on biofilm cells is suggested in future studies.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Naglik JR, Fostira F, Ruprai J, Staab JF, Challacombe SJ, Sundstrom P. *Candida albicans* HWP1 gene expression and host antibody responses in colonization and disease. J Med Microbiol 2006;55:1323-7.
2. Alves CT, Wei XQ, Silva S, Azeredo J, Henriques M, Williams DW. *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. J Infect 2014;69:396-407.
3. Brockert PJ, Lachke SA, Srikantha T, Pujol C, Galask R, Soll DR. Phenotypic switching and mating type switching of *Candida glabrata* at sites of colonization. Infect Immun 2003;71:7109-18.
4. Sun JN, Solis NV, Phan QT, Bajwa JS, Kashleva H, Thompson A, *et al*. Host cell invasion and virulence mediated by *Candida albicans*. PLoS Pathog 2010;6:1-14.
5. Gropp K, Schild L, Schindler S, Hube B, Zipfel PF, Skerka C. The yeast *Candida albicans* evades human complement attack by secretion of aspartic proteases. Mol Immunol 2009;47:465-75.
6. Zhao X, Oh SH, Hoyer LL. Unequal contribution of ALS9 alleles to

adhesion between *Candida albicans* and human vascular endothelial cells. Microbiology 2007;153(Pt 7):2342-50.
7. Hube B, Stehr F, Bossenz M, Mazur A, Kretschmar M, Schäfer W. Secreted lipases of *Candida albicans*: Cloning, characterisation and expression analysis of a new gene family with at least ten members. Arch Microbiol 2000;174:362-74.
8. Hoyer LL, Green CB, Oh SH, Zhao X. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family – A sticky pursuit. Med Mycol 2008;46:1-15.
9. Zhao X, Oh SH, Cheng G, Green CB, Nuessen JA, Yeater K, *et al.* ALS3 and ALS8 represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. Microbiology 2004;150:2415-28.
10. Du H, Guan G, Xie J, Sun Y, Tong Y, Zhang L, *et al.* Roles of *Candida albicans* Gat2, a GATA-type zinc finger transcription factor, in biofilm formation, filamentous growth and virulence. PLoS One 2012;7:e29707.
11. Li F, Svarovsky MJ, Karlsson AJ, Wagner JP, Marchillo K, Oshel P, *et al.* Eap1p, an adhesin that mediates *Candida albicans* biofilm formation *in vitro* and *in vivo*. Eukaryot Cell 2007;6:931-9.
12. Murciano C, Moyes DL, Runglall M, Tobouti P, Islam A, Hoyer LL, *et al.* Evaluation of the role of *Candida albicans* agglutinin-like sequence (Als) proteins in human oral epithelial cell interactions. PLoS One 2012;7:e33362.
13. Hoyer LL, Hecht JE. The ALS5 gene of *Candida albicans* and analysis of the Als5p N-terminal domain. Yeast 2001;18:49-60.
14. Liu Y, Filler SG. *Candida albicans* Als3, a multifunctional adhesin and invasin. Eukaryot Cell 2011;10:168-73.
15. Schaller M, Bein M, Korting HC, Baur S, Hamm G, Monod M, *et al.* The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an *in vitro* model of vaginal candidiasis based on reconstituted human vaginal epithelium. Infect Immun 2003;71:3227-34.
16. Stehr F, Felk A, Gácser A, Kretschmar M, Mähnss B, Neuber K, *et al.* Expression analysis of the *Candida albicans* lipase gene family during experimental infections and in patient samples. FEMS Yeast Res 2004;4:401-8.
17. Roudbary M, Roudbarmohammadi SH, Bakhshi B, Farhadi Z. Relation of ALS 1 and ALS3 genes and fluconazole resistance in *Candida albicans* isolated from vaginal candidiasis. Inter J Mol Clin Microbiol 2012;2:170-4.
18. Ausubel FM, Brent R, Kingston RE, More D. Current Protocols in Molecular Biology. John wiley & sons Inc; ringbou edition 2003.
19. Kavanagh K. New Insight in Medical Mycology. Netherland: Springer; 2007.
20. Cheng G, Wozniak K, Wallig MA, Fidel PL Jr., Trupin SR, Hoyer LL. Comparison between *Candida albicans* agglutinin-like sequence gene expression patterns in human clinical specimens and models of vaginal candidiasis. Infect Immun 2005;73:1656-63.
21. Nas T, Kalkanci A, Fidan I, Hizel K, Bolat S, Yolbakan S, *et al.* Expression of ALS1, HWP1 and SAP4 genes in *Candida albicans* strains isolated from women with vaginitis. Folia Microbiol (Praha) 2008;53:179-83.
22. Dhamgaye S, Bernard M, Lelandais G, Sismeiro O, Lemoine S, Coppée JY, *et al*. RNA sequencing revealed novel actors of the acquisition of drug resistance in *Candida albicans*. BMC Genomics 2012;13:396.
23. García-Sánchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d'Enfert C. *Candida albicans* biofilms: A developmental state associated with specific and stable gene expression patterns. Eukaryot Cell 2004;3:536-45.
24. İnci M, Atalya MA, Ozer B, Evirgen O, Duran N, Nedretkoc A, *et al*. Investigations of ALS1 and HWP1 genes in clinical isolates of *Candida albicans*. Turk J Med Sci 2013;43:125-30.
25. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. J Bacteriol 2001;183:5385-94.
26. Kumamoto CA. Candida biofilms: An update. Eukaryot Cells 2005;4: 633-638.
27. Uwamahoro N, Qu Y, Jelicic B, Lo TL, Beaurepaire C, Bantun F, *et al.* The functions of Mediator in *Candida albicans* support a role in shaping species-specific gene expression. PLoS Genet 2012;8:e1002613.
28. Modrzewska B, Kurnatowski P. Adherence of *Candida* sp. to host tissues and cells as one of its pathogenicity features. Ann Parasitol 2015;61:3-9.
29. Argimón S, Wishart JA, Leng R, Macaskill S, Mavor A, Alexandris T, *et al*. Developmental regulation of an adhesin gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. Eukaryot Cell 2007;6:682-92.
30. Bastidas RJ, Heitman J, Cardenas ME. The protein kinase Tor1 regulates adhesin gene expression in *Candida albicans*. PLoS Pathog 2009;5:e1000294.